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Immortalized cells as experimental models to study cancer

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Abstract

The development of cancer is a multi-step process in which normal cells sustain a series of genetic alterations that together program the malignant phenotype. Much of our knowledge of cancer biology results from the detailed study of specimens and cell lines derived from patient tumors. While these approaches continue to yield critical information regarding the identity, number, and types of alterations found in human tumors, further progress in understanding the molecular basis of malignant transformation depends upon the generation and use of increasingly sophisticated experimental models of cancer. Over the past several years, the recognition that telomeres and telomerase play essential roles in regulating cell lifespan now permits the development of new models of human cancer. Here we review recent progress in the use of immortalized human cells as a foundation for understanding the molecular basis of cancer.

Abbreviations: ALT – alternative lengthening of telomeres; HEK – human embryonic kidney; HMEC – human mammary epithelial cell; HPV – human papillomavirus; hTERT – human telomerase reverse transcriptase; LT – SV40 Large T antigen; PD – population doubling; PP2A – protein phosphatase 2A; RalGEF – Ral guanine nucleotide exchange factor; RAS – activated H-Ras allele; shRNA – short hairpin RNA; ST – SV40 small t antigen; TRF – telomere restriction fragment.

Introduction

The study of specimens and cell lines derived from patients reveals a staggering number of genetic alterations associated with most human cancers. However, studying such tissues does not permit the identification of specific mutations critical for initiating or maintaining the transformed state. The development of experimental models of human cancer represents a complementary approach to understanding the molecular basis of cancer. Here we review how recent progress in our understanding of telomeres and telomerase has facilitated the development of cancer models designed to identify pathways critical for transformation.

Telomeres, telomerase, and hTERT

Telomeres are DNA-protein complexes that constitute and protect chromosomal ends from degradation. Loss of telomeric sequences due to the incomplete replication of chromosomal ends

(Watson 1972) eventually leads to genetic instability. As a consequence, eukaryotic organisms have developed highly effective mechanisms to protect chromosome ends from recognition as broken DNA fragments.

In mammals, telomeres consist of repeated double-stranded 5′-TTAGGG-3′ hexameric sequences (Moyzis et al. 1988). In addition, proteins such as TRF1, TRF2, and POT1 bind directly to telomeric DNA, while other proteins interact with these telomere binding proteins (reviewed by Blackburn 2001). Together, these proteins and telomeric DNA form telomeric heterochromatin. The terminal end of the telomere is comprised of a single-stranded 3′ extension to which proteins such as POT1 bind (Baumann and Cech 2001). Ultrastructural studies predict that this single-stranded end folds to form a duplex loop (Griffith et al. 1999). As reviewed in this issue by Dominique Broccoli, determining the identities and functions of these telomere-associated proteins is an active area of research.

The telomerase holoenzyme is a specialized reverse transcriptase that synthesizes telomeric repeats (Greider and Blackburn 1985; Yu et al. 1990). Human telomerase is comprised of two core components: an RNA subunit (*hTERC*) and a protein catalytic subunit (hTERT). *hTERC* is a non-coding functional molecule that carries the RNA template for telomere synthesis (Greider and Blackburn 1989; Yu et al. 1990). Studies from several laboratories have mapped some of the structural elements of *hTERC* necessary for complex formation with hTERT (reviewed by Collins and Mitchell 2002). *hTERC* is ubiquitously expressed in most human cell lines (Feng et al. 1995).

In contrast, *hTERT* expression is under tight regulatory control and is the limiting component for telomerase catalytic activity (Meyerson et al. 1997; Nakamura et al. 1997). Multiple factors regulate *hTERT* expression at the transcriptional level, including activation by the estrogen receptor and c-Myc and repression by Mad1 and Menin (reviewed by Cech 2004). These and other yet undiscovered regulatory mechanisms create a tight balance of repression and activation of *hTERT* expression. Consequently, most human cells express levels of hTERT that are insufficient to maintain telomere length with successive cycles of cell division (Meyerson et al. 1997; Masutomi et al. 2003). As a result, telomeres shorten with passage in culture (Harley et al. 1990).

Barriers to immortalization: replicative senescence

After a limited number of population doublings (PD) in culture (50–100), primary human cells cease dividing and enter a state termed replicative senescence (or mortality stage 1, M1) (Hayflick and Moorhead 1961). At replicative senescence, cells are metabolically active but do not divide. Hallmarks of replicative senescence include insensitivity to growth factor stimulation (Seshadri and Campisi 1990), staining for acidic β-galactosidase (Dimri et al. 1995) and accumulation of senescence-associated heterochromatic foci (Narita et al. 2003).

The molecular mechanism(s) that induce replicative senescence remain unclear. However, some experimental evidence implicates telomeres as one factor that provokes replicative senescence. Cells passaged *in vitro* display progressively shorter telomeres as they approach replicative senescence (Harley et al. 1990). In addition, cells from older individuals exhibit a more limited replicative capacity and shorter telomere lengths than cells derived from younger individuals (Harley et al. 1990; Hastie et al. 1990). Ectopic expression of hTERT restores constitutive telomerase activity, stabilizes telomeres and directly immortalizes some human cell types including human diploid foreskin fibroblasts, retinal pigment epithelial cells (Bodnar et al. 1998; Vaziri and Benchimol 1998), vascular endothelial cells (Yang et al. 1999), mesothelial cells (Dickson et al. 2000) and osteoblasts (Xiaoxue et al. 2004). These observations support a model in which progressive telomere attrition triggers replicative senescence.

However, it is also clear that several other stimuli can trigger a growth arrest state that shares morphological and biological similarity to replicative senescence. The overexpression of oncogenes such as an activated allele of *H-Ras* (hereafter referred to as *RAS*) (Serrano et al. 1997) or exposure to oxidative DNA damage (Chen et al. 1995) induce states of cell growth arrest similar to replicative senescence. Oxidative damage-induced senescence can occur without appreciable telomere shortening (Gorbunova et al. 2002). Constitutive ectopic hTERT expression allows fibroblasts to

surpass replicative senescence, but not *RAS*-induced senescence (Wei and Sedivy 1999). These observations suggest that other stimuli that trigger a senescence-like state may do so independently of effects on telomere length.

Furthermore, constitutive ectopic hTERT expression is insufficient to immortalize other cell types including keratinocytes, human mammary epithelial cells (HMECs), (Kiyono et al. 1998), airway epithelial cells (Lundberg et al. 2002), and preadipocytes (Darimont et al. 2003) although in some cases, culture conditions permit cell immortalization with the expression of hTERT (Ramirez et al. 2001). Immortal keratinocyte clones that eventually arise after the overexpression of hTERT show evidence of inactivation of the retinoblastoma (RB)/$p16^{INK4A}$ tumor-suppressor pathway (Kiyono et al. 1998; Dickson et al. 2000). A telomere-independent growth arrest (M0) limits the growth of HMECs, but inactivation of the RB/$p16^{INK4A}$ pathway alleviates this proliferative block (Foster and Galloway 1996; Foster et al. 1998). Once the RB/$p16^{INK4A}$ pathway is experimentally inactivated, the constitutive ectopic expression of hTERT suffices to immortalize keratinocytes, HMECs and many other human cell types (Kiyono et al. 1998; Lundberg et al. 2002; Darimont et al. 2003; Kyo et al. 2003). These results suggest that the RB/$p16^{INK4A}$ pathway plays an important role in the activation of senescence.

Although it remains unclear how different stimuli trigger senescence, it is well-established that the p53 and RB tumor suppressor pathways mediate the senescence response in human cells. Experimentally, suppression of these pathways by the introduction of the SV40 DNA tumor virus large T (LT) oncoprotein or the combined expression of human papillomavirus (HPV) E6 and E7 oncoproteins allows cells to bypass replicative senescence (Shay and Wright 1989; Shay et al. 1991). In consonance with these findings, perturbation of both pathways is required to render human cells unable to respond to senescence-inducing stimuli (Serrano et al. 1997; Smogorzewska and de Lange 2002).

Telomere shortening may induce senescence via activation of these tumor suppressor pathways, but this mechanism is not well defined. In endothelial cells, telomere restriction fragment (TRF) lengths at the onset of replicative senescence are ~5.7 kbp (Chang and Harley 1995), yet human umbilical vein endothelial cells immortalized by the expression of hTERT bypass replicative senescence with TRF lengths of 2–2.5 kbp (Yang et al. 1999). These observations suggest that a specific telomere length does not trigger replicative senescence. In support of this hypothesis, changes in telomere state, possibly through the loss of the 3′ overhang, induce senescence in a telomere length-independent fashion (Karlseder et al. 2002; Stewart et al. 2003). Collectively, these data indicate that structural changes at the telomere rather than shortened telomeres correlate with the onset of replicative senescence.

Emerging evidence indicates that uncapped telomeres may contribute to the activation of the DNA damage checkpoint response (d'Adda di Fagagna et al. 2003; Sedelnikova et al. 2004). These observations suggest that the activation of DNA damage repair pathways in response to structural changes at telomere ends triggers the p53 and RB pathways to induce senescence. Although replicative senescence occurs in explanted cells *in vitro*, the involvement of tumor suppressor pathways commonly mutated in human cancer *in vivo* lends support to the idea that replicative senescence may also play a role in tumor growth suppression (reviewed by Campisi 2001).

Barriers to immortalization: crisis

Telomeres shorten with successive passage in culture in cells that bypass replicative senescence after inactivation of the p53 and RB pathways. Without constitutive telomerase expression, telomere attrition eventually triggers cellular crisis (or mortality stage 2, M2). Crisis is telomere length-dependent and occurs when shortened telomeres can no longer protect chromosome ends (Counter et al. 1994). This state leads to genomic instability (Counter et al. 1992) characterized by multiple chromosomal breakage and fusion events (reviewed by Maser and DePinho 2002).

Although most cells die during crisis, rare variants escape at a frequency of approximately 1×10^{-7}. (Shay et al. 1993). Most of these survivors show evidence of hTERT expression and are immortal (Counter et al. 1992). These observations suggest that crisis provides a strong selective pressure for the outgrowth of clones that obtain the ability to maintain telomere length. Consistent

with this notion, the majority of human tumor cells (85–90%) exhibit telomerase activity (Kim et al. 1994; Shay and Bacchetti 1997). Collectively, these findings suggest that the capacity to maintain telomeres is critical for immortalization both *in vitro* and *in vivo*.

While most cells that survive crisis reactivate telomerase, a small but significant percentage of these cells do not. These cells acquire the capacity to maintain stable long telomeres in a telomerase-independent manner, termed 'alternative lengthening of telomeres' or ALT (Bryan et al. 1997). Some human tumors also show evidence of ALT, suggesting that ALT occurs *in vivo* (reviewed by Henson et al. 2002). However, the mechanism(s) responsible for the ALT phenotype in human cells are not fully understood.

One of the hallmarks of cancer cells is the immortalized phenotype (reviewed by Hanahan and Weinberg 2000). Since immortalization is one consequence of surviving crisis, telomeres may serve to limit cell lifespan and to provide a defense against unrestrained proliferation. This model has led some to propose that telomerase inhibition may be an effective anti-neoplastic strategy (Counter et al. 1992). In support of this model, mice lacking both *mTERC* and $p16^{INK4A}$ show a reduced incidence of tumor formation compared with mice lacking $p16^{INK4A}$ alone (Greenberg et al. 1999). This experiment suggests that in certain genetic backgrounds, telomere dysfunction can impair tumorigenesis.

In contrast, mice lacking only *mTERC* develop tumors at accelerated rates after 4–6 generations (Blasco et al. 1997). This effect is amplified in the p53 null background (Chin et al. 1999). These observations suggest an alternative model in which the genomic instability associated with telomere dysfunction can promote malignancy (reviewed by Maser and DePinho 2002). Collectively, these experiments indicate that the contribution of telomeres to tumorigenesis *in vivo* may be more complex than initially appreciated.

Cooperating genetic lesions are required for transformation

Although cancer cells display an immortal phenotype, cells overexpressing hTERT are distinct from cancer cells. Specifically, such immortalized cells do not show disruption of the p53 and RB pathways, respond normally to serum-starvation and contact-inhibition, fail to grow in an anchorage-independent manner (no colony formation in soft agar) and cannot form tumors when placed in immunodeficient hosts (Jiang et al. 1999; Morales et al. 1999). These observations indicate that additional genetic alterations are required to induce malignant growth.

Murine models have been useful in understanding how genetic alterations drive tumorigenesis. The introduction of certain pairs of oncogenes such as *c-Myc* and *RAS* or the adenovirus *E1A* gene and *RAS* permits the *in vitro* transformation of murine embryo fibroblasts (Land et al. 1983; Ruley 1983). The constitutive expression of both *c-Myc* and *RAS* in the germ line of transgenic mice or in urogenital sinus cells used to reconstitute the murine prostate gland accelerates the kinetics of tumor growth beyond that observed with either oncogene alone (Sinn et al. 1987; Thompson et al. 1989). These observations indicate that pairs of genetic lesions cooperate to promote malignancy. However, *c-Myc*/*RAS* double transgenic mice are viable, and monoclonal tumors arise stochastically in both germ-line transgenic and prostate-reconstituted animals (Sinn et al. 1987; Thompson et al. 1989). These observations suggest that, in addition to the introduced transgenes, additional somatic mutation(s) are necessary for full tumor progression.

While more than 2 lesions appear required for the transformation of murine cells, epidemiological evidence suggests that 4–6 genetic lesions are required for the transformation of human cells (Renan 1993). Consistent with these statistical studies is the observation that progression of colon carcinoma occurs in at least 4–6 distinct histopathological stages (Kinzler and Vogelstein 1996) that are each accompanied by certain genetic lesions (Vogelstein et al. 1988). Indeed, while pairs of oncogenes such as *c-Myc* and *RAS* cooperate to transform murine cells (Land et al. 1983), they fail to transform human cells (Stevenson and Volsky 1986).

Differences in telomere biology between murine and human cells may partially account for their differential susceptibility to transformation. Murine cells constitutively express *mTERT* (Prowse and Greider 1995) and maintain telomere lengths that are 3–10 times longer than those in compa-

rable human cells (Kipling and Cooke 1990). Therefore, the extended *in vitro* proliferation of murine cells does not seem to be constrained by telomere length, as is the case in human cells.

The LT and small t (ST) viral oncoproteins of the SV40 early region and *RAS* cooperate with hTERT to transform human fibroblasts, kidney epithelial (HEK) cells (Hahn et al. 1999), astrocytes (Rich et al. 2001), HMECs (Elenbaas et al. 2001), airway epithelial cells (Lundberg et al. 2002), ovarian surface epithelial cells (Liu et al. 2004), mesothelial cells (Yu et al. 2001) and endothelial cells (MacKenzie et al. 2002). Taken together, these observations suggest that multiple mutations cooperate to promote tumorigenesis.

Using defined genetic elements to model specific cancer types

The co-expression of LT, ST, hTERT, and *RAS* is capable of producing transformed human cells that form tumors resembling specific human malignancies. For example, both transformed HMECs and naturally arising breast tumors exhibit chromosomal amplifications containing the *c-Myc* locus (Elenbaas et al. 2001). Moreover, mixing transformed HMECs with Matrigel or stromal cells enhances the efficiency and decreases the latency of orthotopic tumor formation. This experiment suggests that non-cell autonomous factors may play an important role in breast cancer etiology. Indeed, recent evidence that an altered stromal environment can enhance mammary tumor formation supports this hypothesis (Kuperwasser et al. 2004).

Co-expression of this defined set of genetic elements in ovarian epithelial, airway epithelial, and glial cells produces tumorigenic cells that recapitulate multiple features of naturally occurring human cancers when injected into immunocompromised hosts (Rich et al. 2001; Lundberg et al. 2002; Liu et al. 2004). Transformed ovarian epithelial cells form tumors that recapitulate several features of ovarian cancer including similarities in histopathology and dependence upon NF-κB-mediated cytokine signaling for survival (Liu et al. 2004). Similarly, histological similarities exist between tumors arising from experimentally transformed glial or airway epithelial cells and naturally occurring gliomas or squamous cell carcinomas of the lung, respectively (Rich et al. 2001; Lundberg et al. 2002).

These studies suggest that the introduction of LT, ST, hTERT, and *RAS* into primary cells results in experimental models that retain some of the characteristics of the tissues from which the cells originated. However, naturally arising human malignancies do not result from the combined expression of these specific genetic elements. The use of these elements does, however, identify genetic pathways that permit tumor formation. As such, these model systems will help facilitate the study of how particular pathways contribute to human cell transformation.

SV40 LT antigen: inhibiting the RB/p16^{INK4A} and p53/p14^{ARF} pathways

Although viral proteins such as LT and ST do not contribute to the etiology of most human tumors, they promote viral replication by interfering with host cell regulatory pathways that mediate proliferation and survival. Malignant cells frequently exhibit mutations in these same regulatory pathways. Therefore, ectopic expression of viral oncoproteins functionally mimics events that occur in naturally occurring cancer.

LT binds and inactivates the p53 and RB tumor suppressor proteins as well as the RB family members p107 and p130 (Dyson et al. 1989; Zalvide and DeCaprio 1995). Whereas expression of wild-type LT permits human cells to bypass replicative senescence, expression of LT mutants that bind only p53 or RB does not (Shay et al. 1991). Additionally, both the p53- and RB-inactivating capacities of LT are required to allow hTERT-overexpressing cells to avoid *RAS*-induced growth arrest (Hahn et al. 2002).

Similarly, the co-expression of HPV E6 and E7 oncoproteins can substitute for LT in transformation (Hahn et al. 2002) (Figure 1). Co-expression of a dominant-negative allele of p53, *cyclin D1*, plus a mutant allele of *CDK4* (R24C) that renders the kinase insensitive to p16^{INK4A} inhibition also suffices to replace LT in transformation (Hahn et al. 2002). Voorhoeve and Agami (2003) independently confirmed these findings by utilizing short hairpin RNA (shRNA) technology. Specifically, they showed that shRNA-mediated suppression of both RB and p53 expression

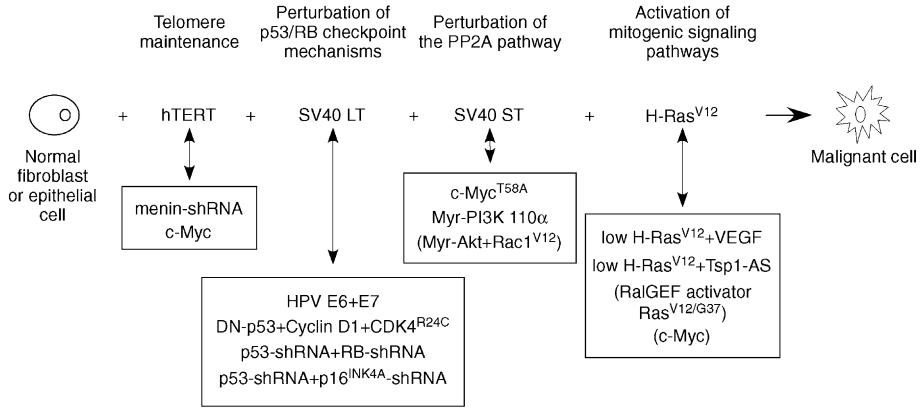

Figure 1. Genetic elements that suffice to transform human cells. Lesions that cooperate to transform multiple types of normal fibroblast or epithelial cell lines are shown. Boxes contain elements that substitute for hTERT, LT, ST, or *RAS* expression. Although multiple regulatory mechanisms exist to regulate hTERT and *RAS*.expression, only those directly tested in transformation models are shown. Alterations that induce anchorage-independent growth but not tumor formation in immunocompromised hosts are in parentheses. Abbreviations used: AS: antisense; DN: dominant negative; HPV: human papillomavirus; LT: SV40 large T antigen; Myr: myristoylated; PI3K: phosphatidylinositol 3-kinase; PP2A: protein phosphatase 2A; RalGEF: Ral guanine nucleotide exchange factor; shRNA; gene specific short hairpin RNA; ST: SV40 small t antigen; VEGF: vascular endothelial growth factor.

cooperates with hTERT, ST, and *RAS* to transform BJ fibroblasts (Figure 1). These results suggest that inactivation of both the p53 and RB tumor suppressor pathways cooperate with hTERT, ST, and *RAS* to render cells tumorigenic. In agreement with these observations, most human tumors contain mutations that perturb the p53 and RB pathways.

The *INK4A* locus encodes two tumor-suppressor genes that regulate the RB and p53 pathways: p16^{INK4A} and p14^{ARF} (p19^{ARF} in mice) (reviewed by Lowe and Sherr 2003). Expression of a p16^{INK4A}-specific shRNA can substitute for expression of an RB-specific shRNA to achieve transformation of BJ cells (Voorhoeve and Agami 2003). However, expression of a p14^{ARF}-specific shRNA fails to substitute for expression of a p53-specific shRNA in transformation when co-expressed with a p16^{INK4A}-specific shRNA or an RB-specific shRNA, plus hTERT, ST, and *RAS*. These results suggest that loss of p16^{INK4A} substitutes for loss of RB in human cell transformation.

In contrast, Wei et al. (2003) addressed this issue by expressing a cyclin D1-CDK4^{R24C} fusion protein in LF1 lung fibroblasts. Expression of the cyclin D1-CDK4^{R24C} fusion protein renders the cells insensitive to p16^{INK4A}, yet fails to cooperate with the co-expression of hTERT, ST, and *RAS* to transform a p53 null LF1 line. However, expression of LT transforms wild-type LF1 cells expressing hTERT, ST, and *RAS*. These experiments suggest that loss of p53 and p16^{INK4A} function is not equivalent to LT expression in transformation (Wei et al. 2003). Differences between the cell lines used and/or the methods of p53 and RB pathway perturbation may, in part, explain these divergent conclusions. Indeed, certain lines of human fibroblasts are more susceptible to transformation than others (Akagi et al. 2003). In addition, differential levels of p16^{INK4A} expression between fibroblast strains mediate susceptibility to *RAS*-induced growth arrest (Benanti and Galloway 2004).

A complementary approach to understanding the role of p16^{INK4A} in suppressing transformation is the use of p16^{INK4A}-deficient fibroblasts (Leiden cells) (Brookes et al. 2002; Drayton et al. 2003). These human cells have a homozygous deletion in *INK4A* that renders them 16^{INK4A} deficient, yet they translate a protein that retains some of the known functions of p14^{ARF} (Brookes et al. 2002). Co-expression of hTERT, *c-Myc*, and *RAS* in Leiden cells, but not in control p16^{INK4A}-expressing fibroblasts, allows for tumor formation in immunocompromised mice, but at long latency and low frequency (Drayton et al. 2003). These observations suggest that additional unknown lesion(s) may have spontaneously occurred during tumor formation (Drayton et al. 2003) and

support previous findings delineating the importance of the $p16^{INK4A}$ tumor suppressor protein in transformation (Sharpless et al. 2001; Voorhoeve and Agami 2003).

Since many factors contribute to the regulation of the RB pathway, further work is necessary to understand the contribution of these various members of the RB pathway to human cell transformation. Thus, careful consideration of the manner that the RB/$p16^{INK4A}$ pathway is perturbed may be critical in ascertaining how it contributes to tumor-suppression.

SV40 ST antigen: perturbing protein phosphatase 2A

Alternative splicing of the transcript encoded by the SV40 early region produces an additional oncoprotein, ST. Expression of ST facilitates the transformation of cells by LT (Bikel et al. 1987). Infection of established human cell lines with wild-type SV40 virus induces focus formation, but infection with mutant SV40 viruses that do not produce ST fails to do so (de Ronde et al. 1989). In consonance with these observations, expression of LT alone fails to transform human cells expressing hTERT and *RAS* (Hahn et al. 2002). The further introduction of ST into these cells rescues the transformation phenotype.

Although LT and ST share 82 amino acids at their N-terminus, ST has a unique C-terminus that binds and inactivates the protein phosphatase 2A (PP2A) family of serine/threonine phosphatases (Pallas et al. 1990; Yang et al. 1991). Mutations that render ST unable to inhibit PP2A are greatly reduced in their ability to transform growth-arrested rat F111 cells (Mungre et al. 1994). ST mutant alleles that retain the capacity to bind PP2A transform human cells expressing hTERT, LT, and *RAS*, yet mutant alleles that cannot bind PP2A are unable to transform such cells (Hahn et al. 2002). In addition, inhibition of PP2A by okadaic acid treatment substitutes for ST in transformation (Chen et al. 2004). These observations indicate that disruption of PP2A function is required for transformation of human cells.

PP2A is an ubiquitously expressed enzymatic complex consisting of multiple subunits (reviewed by Lechward et al. 2001). Due to the structural and functional complexity of PP2A, the critical cellular target(s) of PP2A perturbed by ST expression remain undefined. However, recent studies provide genetic evidence for the involvement of the phosphatidylinositol 3-kinase (PI3K) and *c-Myc* signaling pathways in ST-induced transformation (Zhao et al. 2003; Yeh et al. 2004). Late passage HMECs that express hTERT and LT do not require *RAS* expression for anchorage-independent growth, and ST expression alone transforms these cells (Zhao et al. 2003). Since *RAS* expression activates multiple signaling pathways (reviewed by Malumbres and Barbacid 2003) and is typically required for transformation of HMECs (Elenbaas et al. 2001), late passage HMECs provide an opportunity to identify ST-specific targets without the confounding effects of *RAS* expression. In these cells, expression of a constitutively-active PI3K allele (myr-p110α) functionally replaces ST in transformation (Zhao et al. 2003) (Figure 1). Furthermore, the combined effects of constitutively activated *Akt1* and *Rac1* substitute for myr-p110α.

Recent evidence also suggests that ST suppresses the degradation of c-Myc (Yeh et al. 2004). Dephosphorylation of serine 62 of c-Myc decreases its stability, while dephosphorylation of serine 58 increases c-Myc stability (Sears et al. 2000). *In vitro*, purified PP2A desphosphorylates serine 62 of adenoviral-expressed c-Myc from quiescent REF52 cells (Yeh et al. 2004). Inhibition of PP2A by okadaic acid treatment or ST expression promotes c-Myc accumulation and stability. Finally, a stabilized allele of c-Myc (T58A) can replace ST in rendering hTERT/LT/*RAS*-expressing HEK cells tumorigenic (Yeh et al. 2004) (Figure 1).

Together, these observations provide genetic evidence for the involvement of the PI3K and *c-Myc* signaling pathways in transformation by ST (Zhao et al. 2003; Yeh et al. 2004). However, it is currently unclear which PP2A target(s) contribute to the observed phenotypes and if human tumors contain mutations in these molecules. Identifying the specific target(s) of PP2A involved in human cell transformation is an active area of research.

hTERT

As discussed above, the capacity to maintain telomeres is required for the immortalization of multiple cell types. Further, 85–90% of human

tumors show evidence of hTERT catalytic activity (Kim et al. 1994; Shay and Bacchetti 1997). Co-expression of LT, ST, and *RAS* fails to transform multiple types of human cells without the concomitant expression of hTERT (Hahn et al. 1999; Elenbaas et al. 2001) or the de-repression of *hTERT* expression by the expression of a Menin-specific shRNA (Lin and Elledge 2003) (Figure 1). These findings support the hypothesis that telomerase activity is necessary for human cell transformation.

Recent results, however, suggest that telomerase activity is not an obligate requirement for initial tumor formation under certain circumstances (Seger et al. 2002). The co-expression of the adenovirus E1A oncoprotein and *RAS* suffices to induce anchorage-independent growth of BJ cells in soft agar. These cells, however, are not tumorigenic. By overexpressing *MDM2*, Seger et al. showed that the further inactivation of p53 renders these cells capable of tumorigenic growth. Both E1A/*RAS*/*MDM2*-expressing BJ cells and lysates from tumor tissues derived from these cells lack telomerase activity. However, these cells undergo crisis either after extended passaging *in vitro* (40–50 PDs) or after explantation into culture. Cells which survive this period of crisis show telomerase activity (Seger et al. 2002).

Primary human keratinocytes that co-express *RAS* and *CDK4* do not have elevated hTERT protein levels and are not immortalized (Lazarov et al. 2002). However, *in vivo* co-expression of *RAS* and *CDK4* in keratinocytes using multiplex serial gene transfer induces the formation of squamous cell carcinomas. These tumors display maintenance of telomere length and increased expression of hTERT protein (Lazarov et al. 2002). Collectively, these findings indicate that the capacity to maintain telomeres is ultimately required during tumorigenesis, although cells may not require telomerase activity for initial tumor formation.

H-Ras^{V12}

RAS induces multiple downstream pathways, each of which may participate in cancer development. Three major *RAS* effector pathways implicated in tumor formation are the Raf pathway, the PI3K pathway, and the Ral guanine nucleotide exchange factor (RalGEF) pathway (reviewed by Malumbres and Barbacid 2003). Since many molecules have been implicated in *RAS* signaling, it is difficult to identify the particular effectors that mediate the transforming capacity of *RAS*. However, Hamad et al. and Rangarajan et al. used individual *H-Ras*^{V12} mutant alleles (T35S, Y40C, E37G) that each activate one of these downstream pathways (Raf, PI3K, RalGEF, respectively) to delineate the roles of *RAS*-effector pathways in tumorigenesis (Hamad et al. 2002; Rangarajan et al. 2004). In HEK cells and human astrocytes, expression of *RAS*^{E37G} cooperates with hTERT, LT, and ST to induce growth in soft agar, yet expression of *RAS*^{T35S} or *RAS*^{Y40C} fails to do so (Hamad et al. 2002) (Figure 1). This result implicates the RalGEF pathway in *RAS*-induced transformation of human cells *in vitro*. However, HEK cells transformed by the co-expression of hTERT, LT, ST, and *RAS*^{E37G} fail to form tumors in nude mice. The additional co-expression of *RAS*^{T35S} and *RAS*^{Y40C} renders such cells tumorigenic (Hamad et al. 2002), suggesting that the combined effects of the Raf, PI3K and RalGEF pathways together mediate *RAS*-induced tumorigenesis *in vivo*. Indeed, different pathways downstream of RAS mediate the transformation of different human cell types (Rangarajan et al. 2004).

Recent reports implicate the *c-Myc* oncoprotein as one downstream target of *RAS* that may partially mediate *RAS*-induced tumorigenesis (Sears et al. 2000; Wei et al. 2003; Zhao et al. 2003). *RAS* expression enhances c-Myc stability in quiescent REF52 rodent cells and the activities of the Raf and PI3K pathways mediate this effect (Sears et al. 2000). Elevated *c-Myc* expression can substitute for *RAS* expression in the transformation of HMECs expressing hTERT, LT and PI3K myr-p110α (Zhao et al. 2003). *c-Myc* expression can also replace *RAS* expression in the transformation of LF1 cells expressing hTERT, LT, and ST (Wei et al. 2003) (Figure 1). Collectively, these results suggest that *c-Myc* may play a role in *RAS*-induced tumorigenesis, but it is unclear which effector pathways mediate this effect in human cells.

Transformation of HMECs directly correlates with the relative level of *RAS* overexpression (Elenbaas et al. 2001). Whereas HMECs that co-express hTERT, LT, ST, and high levels of *RAS* are tumorigenic, HMECs that express more modest levels of *RAS* are not. Overexpression of

vascular endothelial growth factor (VEGF) or antisense suppression of thrombospondin-1 (*Tsp1*) renders these low *RAS*-expressing cells capable of tumor formation (Watnick et al. 2003) (Figure 1). These results suggest that a deficiency in angiogenic potential may limit the tumorigenicity of low *RAS*-expressing cells. Thus, one important function of *RAS* in tumorigenesis may be to promote vascular recruitment.

Taken together, these experiments highlight the complexity of *RAS* signaling in human tumorigenesis. Multiple effector arms of *RAS* signaling are involved in transformation, yet the specific requirements for activation of these pathways may differ between cell types. Indeed, the results of Hamad et al. and Rangarajan et al. indicate that different *RAS* effector pathways participate in human and murine cell transformation (Hamad et al. 2002; Rangarajan et al. 2004). It is additionally unclear why the *RAS* expression levels required for tumorigenesis in these types of experiments is higher than those found in human tumors. Further studies are necessary to clarify which proteins operate downstream of *RAS* in malignancy.

Concluding remarks

The limited replicative lifespan of cultured human cells is often a limiting factor in building cancer models. Due to their capacity to surpass replicative barriers in culture, immortalized cells of various types constitute robust model systems with which to study additional requirements for human cell transformation. Data derived from *in vitro* models of cancer effectively complements data from *in vivo* mouse models of cancer, as well as from studies of tumor specimens derived from patients. The ability to rapidly manipulate the expression of oncogenes and tumor-suppressor genes in *in vitro* models facilitates the identification of genetic lesions that cooperate to program the malignant state.

Human and murine cells have differential susceptibilities to transformation, and differences in telomere biology may partially account for this observation. hTERT overexpression in human cells phenocopies the high-levels of endogenous *mTERT* that murine cells express. Therefore, human cells immortalized by hTERT enable the identification of additional genetic pathways that may differentially regulate human and murine transformation. A full understanding of these species-specific differences in transformation will permit one to rationalize differences between human and mouse cancer models.

Defined sets of genetic elements transform hTERT-immortalized cells. These findings enable studies of genetic lesions which functionally replace one another in tumorigenesis. Unbiased screening approaches using cDNA or shRNA expression libraries in cell lines lacking one or more of these elements (such as hTERT/LT/ST/*RAS*) now represent exciting new strategies for identifying entire classes of genes which cooperate in transfomation. These genomic screens may uncover novel oncogenes or tumor-suppressor genes involved in human malignancy. Alternatively, these approaches may assign new functions to genes not previously known to be involved in tumorigenesis. Through the use of high-throughput methods we may soon realize the full potential of using immortalized cells as model systems of cancer.

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